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## Antibodies to Conserved Pneumococcal Antigens Correlate with, but Are Not Required for, Protection against Pneumococcal Colonization Induced by Prior Exposure in a Mouse Model

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**In mice following intranasal exposure to *Streptococcus pneumoniae*, protection against pneumococcal colonization was independent of antibody but dependent on CD4<sup>+</sup> T cells. Nonetheless, concentrations of antibodies to three conserved pneumococcal antigens correlated with protection against colonization. Concentrations of antibodies to conserved pneumococcal antigens may be correlates of protection without being effectors of protection.**

Antibodies to certain noncapsular antigens arise early in life (8, 18) in response to colonization with, or otitis media caused by, pneumococci (18). This finding has stimulated interest in determining whether such antibodies are correlates of natural protection against colonization or disease; observational studies to assess such correlates are under way. In a study on adult volunteers, antibodies to a fragment of pneumococcal surface protein A (PspA), a surface-exposed protein of *Streptococcus pneumoniae*, correlated with protection against colonization with a serotype 23F pneumococcus, while antibodies against type 23F capsular polysaccharide did not (15).

A correlation between antibodies to a conserved pneumococcal antigen and protection against colonization may occur for either (or both) of two reasons, apart from chance. These antibodies may be effectors of protection, and they may also be markers of host immune responses to prior pneumococcal exposure, which may have induced antibodies to other antigens and/or immunity mediated through other mechanisms. In mice, clearance rates of pneumococcal colonization are similar in wild-type and in  $\mu$ MT mice, which are deficient in antibody production, suggesting that the mechanism of clearance is antibody independent (16). Our previous experiments showed that intranasal exposure to either live *S. pneumoniae* or killed unencapsulated *S. pneumoniae* ("whole-cell vaccine") with cholera toxin adjuvant (WCV/CT) can confer protection against subsequent pneumococcal challenge in  $\mu$ MT mice (13). However, our study did not address whether antibodies induced by exposure to live bacteria played an additional role in protection, augmenting the antibody-independent effect.

The present study had two main objectives. First, we sought to further characterize the mechanisms of protection conferred by exposure to live pneumococci. Specifically, we wished to determine whether protection against colonization following exposure to live pneumococci was entirely independent of an-

tibody and whether it was dependent on CD4<sup>+</sup> T cells, as was the case with WCV/CT (13). Given the substantial differences between WCV/CT and live exposure—notably the use of ethanol killing and the presence of 100 times more bacteria and a potent adjuvant in WCV/CT—we thought it important to determine whether the mechanisms of protection were similar. Second, we sought to determine whether antibodies to conserved pneumococcal antigens were correlated with protection against colonization following intranasal exposure.

To address the first question, we exposed seven groups of 16 mice to approximately  $2 \times 10^6$  CFU of strain TIGR4:14<sup>4</sup>, a serotype 14 variant of the genomically sequenced strain TIGR4 (19) that we had previously constructed (20) and used in similar experiments (13), or to saline as a control (Fig. 1). Groups 1 to 4 were wild type, C57BL/6J, while groups 5 to 7 were  $\mu$ MT<sup>-/-</sup> (B6.129S2-Igh-6tm1Cgn/J) mice in which B-cell development was blocked at the pro-B stage (7). All mice were male, were obtained from Jackson Laboratories, and were 6 weeks old at the start of the experiment. Groups 1 and 5 received saline, while all other groups were exposed to TIGR4:14<sup>4</sup>. These exposures occurred at weeks 0, 1, and 2. On week 3, all groups received 1 mg of rifampin intraperitoneally twice, 1 day apart; this dose (given either once or twice) had been shown previously to eliminate pneumococcal colonization (11). On week 12, all groups were challenged with  $1 \times 10^6$  CFU of strain TIGR4:7F<sup>4</sup>, a serotype 7F variant of TIGR4 that was constructed in a fashion similar to that of TIGR4:14<sup>4</sup>; this variant was isogenic to TIGR4:14<sup>4</sup> apart from the capsular biosynthesis locus (20). Groups 3 and 6 received 1-mg doses of rat anti-mouse CD4 monoclonal immunoglobulin G2b (IgG2b) (purified from hybridoma GK1.5; American Type Culture Collection) 1 day before, 1 day after, and 3 days after TIGR4:7F<sup>4</sup> challenge. As a control for the effect of such a monoclonal antibody, group 4 received the same dose of rat anti-mouse CD8 monoclonal IgG2a (purified from hybridoma 53-6.72; American Type Culture Collection) on the same schedule. Seven days after challenge, all mice were sacrificed and assessed for intranasal colonization by use of a retrotra-

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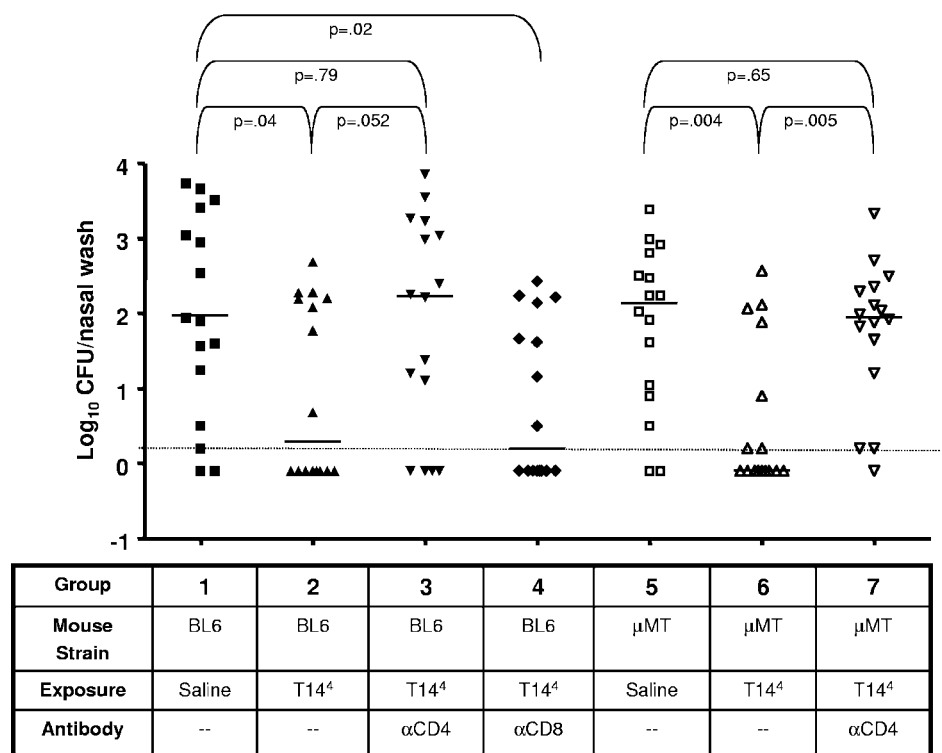


FIG. 1. Intranasal colonization with *S. pneumoniae* TIGR4:7F<sup>4</sup> in normal (C57BL/6/J [BL6]) and antibody-deficient (μMT) mice previously exposed to saline or *S. pneumoniae* TIGR4:14<sup>4</sup> (T14<sup>4</sup>), treated with rifampin, and directly challenged (groups 1 and 2 and groups 5 and 6) or challenged after depletion of CD4<sup>+</sup> cells (groups 3 and 7) or CD8<sup>+</sup> cells (group 4). A dashed line indicates the limit of detection; solid lines indicate group medians. *P* values refer to Mann-Whitney tests for differences in the distributions of CFU/nasal wash between groups.

cheal wash and plating of serial dilutions on gentamicin-containing blood agar plates, as previously described (9, 13). Over 95% depletion of CD4<sup>+</sup> cells from the spleens of mice in groups 3 and 6 and of CD8<sup>+</sup> cells from mice in group 4 was confirmed by flow cytometry. The treatments for each group, as well as the levels of colonization observed, are shown in Fig. 1. The intensities of colonization between groups were compared by the Mann-Whitney U test.

Prior exposure to TIGR4:14<sup>4</sup> reduced the proportion of wild-type mice colonized by TIGR4:7F<sup>4</sup> by 43% (group 1 versus 2), similar to previous findings, and the median intensity of colonization was reduced by 1.5 log<sub>10</sub>. The same pattern was observed in μMT mice, with a 50% reduction in the proportion of animals colonized (group 5 versus 6) and a 2.2 log<sub>10</sub> reduction in the median number of CFU/nasal wash. In both wild-type (group 3 versus 1) and μMT (group 7 versus 5) mice, colonization in CD4<sup>+</sup>-cell-depleted, previously exposed mice was equivalent to that in saline-exposed mice; in other words, all protection conferred by prior exposure was lost in CD4<sup>+</sup>-cell-depleted mice. CD8<sup>+</sup> cell depletion, by contrast, did not affect protection due to prior colonization (group 2 versus 4). We conclude from these results that protection against intranasal colonization in this mouse model is entirely independent of antibody against noncapsular antigens and entirely dependent on the presence of CD4<sup>+</sup> cells at the time of challenge.

Given this finding, we sought to determine whether antibodies to pneumococcal antigens were correlates of protection following pneumococcal exposure, even though they were not

effectors of such protection. To do so, we measured antibodies in the sera of 121 mice that had been exposed to either TIGR4:7F<sup>4</sup>, TIGR4:14<sup>4</sup>, or a similar type 6B variant, TIGR4:6B<sup>4</sup>, and subsequently challenged the mice with one of these three strains in a protocol similar to the one described here, except that the interval between rifampin treatment and challenge was 2 weeks. These were the same mice whose colonization status was previously described (13). Sera were obtained by retro-orbital bleed the day before challenge. Depending on the strains used for initial exposure and for challenge, we previously showed that prior exposure conferred between 24% and 93% protection against colonization (13). Since anticapsular antibodies were not produced in substantial quantities following such colonization (13), we chose to measure IgG antibodies to PspA and pneumococcal surface adhesin A (PsaA). Both antigens have received consideration recently as vaccine candidates. Because little IgG against the pneumococcal cell wall polysaccharide (CWPS) was detected, the total Ig to this antigen was measured.

Enzyme-linked immunosorbent assay (ELISA) plates were coated with 2.5 μg/ml PsaA (Ed Ades, Centers for Disease Control and Prevention, Atlanta, GA), 1 μg/ml PspA (Susan Hollingshead, University of Alabama, Birmingham), or 5 μg/ml CWPS (Statens Serum Institut, Copenhagen, Denmark) in phosphate-buffered saline. Coated plates were washed with phosphate-buffered saline–0.05% Tween 20 (PT) and blocked for 1 h with PT containing 5% fetal calf serum (PTFCS). Dilutions of sera in PT were added for 1 to 2 h, the plates were

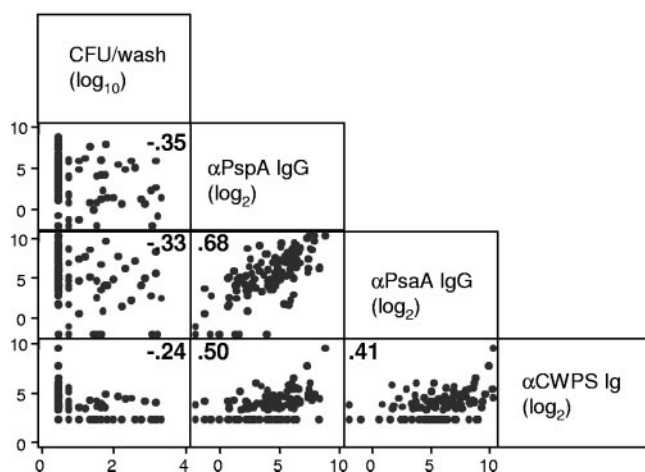


FIG. 2. Relationship between colonization intensities (expressed as  $\log_{10}$  CFU/nasal wash) and antibodies to pneumococcal surface protein A (PspA IgG), pneumococcal surface adhesin A (PsaA IgG), and cell wall polysaccharide (CWPS Ig) (all expressed as  $\log_2$  ELISA units/ml). Correlation coefficients shown in the upper corners of each plot are all statistically significant at a  $P$  value of 0.01 or less.

washed, and peroxidase-conjugated goat antibody to mouse Ig or IgG (Southern Biotechnology, Birmingham, AL) in PT was added for an additional 1 to 2 h. The plates were washed and developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sureblue TMB; KPL, Gaithersburg, MD). Antibody levels were evaluated by parallel titration in comparison to in-house standards assigned an arbitrary value of 100 ELISA units per ml by using SOFTmax PRO (Molecular Devices, Sunnyvale, CA). The in-house standards consisted of pooled serum from mice immunized by intraperitoneal injection with 23-valent polysaccharide vaccine (Pneumovax; Merck, Whitehouse Station, NJ) in MPL adjuvant (RIBI Immunochem, Hamilton, MT) by immunization with WCV/CT and/or by colonization with TIGR4 variants.

Animals were considered colonized for categorical analyses if any pneumococcal colonies were obtained from the nasal wash; for continuous analyses, animals yielding no colonies were assigned values of 0.8 CFU/nasal wash, i.e., one-half of the lower limit of detection. ELISA units were expressed as base-2 logarithms for display and analysis, while numbers of CFU/nasal wash were displayed on a base-10 logarithmic scale. Scatter plots were constructed to show the relationships between concentrations of antibodies to each of the antigens and densities of colonization. Associations between levels of individual antibodies and colonization were assessed by conditional logistic regression, with the antibody as the independent variable and the identity of the challenge strain as the conditioning variable. The latter choice was made because we had observed that different challenge strains had different colonization efficiencies (13). To determine whether any univariate associations between antibody concentrations and protection levels reflected independent associations or simply reflected correlations in different antibody levels across individuals, a multivariate analysis was performed, beginning with a conditional logistic regression model including all three antibody concentrations and removing the antibody concentration pro-

TABLE 1. Odds ratios for colonization for each twofold increase in concentration of antibody against PspA, PsaA, or CWPS<sup>a</sup>

Antigen	Univariate OR (95% CI) <sup>b</sup>	$P$ value
PspA	0.76 (0.64, 0.91)	0.004
PsaA	0.82 (0.70, 0.96)	0.016
CWPS	0.63 (0.44, 0.91)	0.005

<sup>a</sup> Colonization was indicated by the presence of  $\geq 1$  CFU detectable in nasal wash fluid. Data were obtained from conditional logistic regression analysis and grouped by challenge strain. In a multivariate model, only PspA data remained significant.

<sup>b</sup> OR, odds ratio; CI, confidence interval.

ducing the highest  $P$  value until all remaining antibody concentrations produced  $P$  values of less than 0.05 (backwards selection [6]).

Figure 2 shows the relationships between antibody concentrations and densities of colonization in the 121 mice. Each of the antibodies showed a significant correlation with the others, particularly PspA and PsaA, as was previously observed in humans (8, 18). Table 1 and Fig. 2 also show a statistically significant negative relationship between colonization densities and concentrations of antibody to each of the antigens considered separately. Using multivariate regression, we found that these univariate associations mainly reflected correlations in the concentrations of different antibodies (mice with high concentrations of one antibody tended to have high concentrations of the others). The strongest independent association with protection was the concentration of anti-PspA. While this correlation was strongly statistically significant, Fig. 2 and Table 1 show that the relationship was "noisy" (some highly colonized animals had high antibody concentrations, and some uncolonized animals had undetectable concentrations). These findings are consistent with the hypothesis that the concentration of each of these antibodies is an imperfect marker of exposure.

Taken together, these results indicate that in C57BL/6J mice, colonization 9 weeks previously with *S. pneumoniae* can confer significant (although not complete) protection against subsequent colonization events through a mechanism that is independent of antibodies and dependent on CD4<sup>+</sup> T cells present at the time of challenge. However, each of the antibodies we chose to measure is a statistically significant correlate of protection in such mice (Table 1 and Fig. 2), and one of them, PspA, has been observed to be a correlate of protection in humans (15). Together, our results suggest that these observed antibody correlates of protection may be indicators of the intensity of the immune response mounted after prior exposure, even if the antibodies themselves are not the mechanistic effectors of protection.

We have focused this study solely on "naturally acquired" mechanisms of immunity to colonization. The mechanisms of protection against invasive pneumococcal disease (10) may be quite different from those involved in colonization. For example, human antibodies to phosphorylcholine, a component of CWPS, can protect mice against challenge with a transparent-phase serotype 6A strain of *S. pneumoniae* (5). Our prior studies have shown that the serum of WCV/CT-immunized rats can confer passive protection against pneumonia from a serotype 3 strain when the challenge dose of that strain is incubated with the serum prior to challenge (11).



Likewise, in contrast to the protection observed after “natural” exposure to live whole pneumococci, antibodies are certainly the mechanisms of protection elicited by existing polysaccharide and conjugate vaccines, and antibodies are very likely important mechanisms of protection of protein subunit vaccines as well. Anticapsular antibodies elicited by vaccination can protect animals (12) and humans (3, 4, 14) against colonization by the appropriate serotypes, and certain protein antigens administered systemically (17) or intranasally (1, 2) can protect mice against colonization, by a mechanism that is assumed to be antibody dependent. In summary, our experiments demonstrate that antibodies to noncapsular antigens are not involved in, and therefore not necessary for, protection following exposure to live pneumococci. Our results do not deny the possibility that such antibodies may be sufficient for protection against disease or against colonization following other immunizing events, particularly vaccination with immunogenic pneumococcal components. The route of administration of most subunit vaccines (systemic) is different from that utilized here and in our earlier study of intranasal, whole-cell vaccination (13), and antibody levels elicited by subunit vaccines may be considerably higher than those elicited here.

Understanding the mechanisms of natural immunity to pneumococcal colonization and disease is crucial for the development of vaccines based on noncapsular antigens, which are conserved across *S. pneumoniae*. Such vaccines could offer significant advantages over existing, highly effective capsular polysaccharide-conjugate vaccines, including lower price, greater ease of manufacturing, and broader coverage of the diverse serotypes of pneumococci responsible for disease around the world. As experience with the capsular polysaccharide conjugate vaccine in the United States has shown, protection against colonization may dramatically enhance the public health benefit of such vaccines, thanks to the indirect protection offered by herd immunity to colonization. Our results suggest that T-cell-mediated immunity to colonization should be considered in studies of such natural immunity and perhaps also when the effects of candidate vaccines are being evaluated (13). Moreover, care should be taken to distinguish correlates of protection from mechanistic effectors of such protection.

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